



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Phosphoinositide 3-kinase gamma gene knockout impairs postischemic neovascularization and endothelial progenitor cell functions

Citation for published version:

Madeddu, P, Kraenkel, N, Barcelos, LS, Siragusa, M, Campagnolo, P, Oikawa, A, Caporali, A, Herman, A, Azzolino, O, Barberis, L, Perino, A, Damilano, F, Emanuelli, C & Hirsch, E 2008, 'Phosphoinositide 3-kinase gamma gene knockout impairs postischemic neovascularization and endothelial progenitor cell functions', *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 1, pp. 68-76.
<https://doi.org/10.1161/ATVBAHA.107.145573>

Digital Object Identifier (DOI):

[10.1161/ATVBAHA.107.145573](https://doi.org/10.1161/ATVBAHA.107.145573)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Arteriosclerosis, Thrombosis, and Vascular Biology

Publisher Rights Statement:

Copyright © 2008 by American Heart Association, Inc. All rights reserved

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2008 January ; 28(1): 68–76. doi:10.1161/ATVBAHA.107.145573.

Phosphoinositide 3-Kinase γ Gene Knockout Impairs Postischemic Neovascularization and Endothelial Progenitor Cell Functions

Paolo Madeddu, Nicolle Kraenkel, Luciola S. Barcelos, Mauro Siragusa, Paola Campagnolo, Atsuhiko Oikawa, Andrea Caporali, Andrew Herman, Ornella Azzolino, Laura Barberis, Alessia Perino, Federico Damilano, Costanza Emanueli, and Emilio Hirsch
Bristol Heart Institute (P.M., N.K., L.S.B., M.S., P.C., A.O., A.C., C.E.) and Cellular and Molecular Medicine (A.H.), University of Bristol, UK; the Molecular Biotechnology Center (O.A., L.B., A.P., F.D., E.H.), University of Turin, Italy; and INBB (N.K., A.O.), Osilo/Rome, Italy

Abstract

Objective—We evaluated whether phosphatidylinositol 3-kinase γ (PI3K γ) plays a role in reparative neovascularization and endothelial progenitor cell (EPC) function.

Methods and Results—Unilateral limb ischemia was induced in mice lacking the *PI3K γ* gene (*PI3K γ ^{−/−}*) or expressing a catalytically inactive mutant (*PI3K γ ^{KD/KD}*) and wild-type controls (WT). Capillarization and arteriogenesis were reduced in *PI3K γ ^{−/−}* ischemic muscles resulting in delayed reperfusion compared with WT, whereas reparative neovascularization was preserved in *PI3K γ ^{KD/KD}*. In *PI3K γ ^{−/−}* muscles, endothelial cell proliferation was reduced, apoptosis was increased, and interstitial space was infiltrated with leukocytes but lacked cKit⁺ progenitor cells that in WT muscles typically surrounded arterioles. PI3K γ is constitutively expressed by WT EPCs, with expression levels being upregulated by hypoxia. *PI3K γ ^{−/−}* EPCs showed a defect in proliferation, survival, integration into endothelial networks, and migration toward SDF-1. The dysfunctional phenotype was associated with nuclear constraining of FOXO1, reduced Akt and eNOS phosphorylation, and decreased nitric oxide (NO) production. Pretreatment with an NO donor corrected the migratory defect of *PI3K γ ^{−/−}* EPCs. *PI3K γ ^{KD/KD}* EPCs showed reduced Akt phosphorylation, but constitutive activation of eNOS and preserved proliferation, survival, and migration.

Conclusions—We newly demonstrated that PI3K γ modulates angiogenesis, arteriogenesis, and vasculogenesis by mechanisms independent from its kinase activity.

Keywords

limb ischemia; angiogenesis; vasculogenesis; endothelial progenitor cells; migration

Postnatal reparative neovascularization is accomplished by capillary angiogenesis, arteriogenesis, and remodeling of preexisting arteriolar shunts into collateral conductance vessels.¹ Vasculogenesis, which is brought about by endothelial progenitor cells (EPC)

recruited from the bone marrow (BM) or local pools, complements the regenerative potential of resident vascular cells.²

Phosphatidylinositol 3-kinases (PI3Ks) play a crucial role in the regulation of all the above processes. On receptor activation, PI3Ks generate phosphatidylinositol-3,4,5- P_3 (PIP₃), which forms a docking platform for the pleckstrin homology domains of protein kinase B (PKB/Akt) and its activator 3-phosphoinositide-dependent protein kinase 1 (PDK1).³ Akt exerts proangiogenic and vascular prosurvival actions through phosphorylation/activation of endothelial nitric oxide synthase (eNOS),⁴⁻⁷ and inactivation of forkhead box O (FOXO) transcription factors.⁸ In addition, the PI3K-Akt-eNOS axis is crucial for the mobilization of EPCs from BM and their migration to sites of ischemia.^{9,10} Negative control of this pathway is ensured by phosphatases. The phosphatase and tensin homolog on chromosome ten (PTEN) reconverts PIP₃ into PIP₂. Moreover, protein phosphatase 2A (PP2A) dephosphorylates/inactivates eNOS.¹¹

Through the generation of selective inhibitors and gene knockout models, specialized functions of different PI3Ks have been identified.¹² Class I PI3Ks comprise 4 different isoforms, all being expressed by endothelial cells (ECs). The 3 members of the subclass IA (PI3K α , β , and δ) are activated by receptor tyrosine kinases. PI3K γ , the unique member of class IB, is activated by G protein-coupled receptors (GPCR) via binding of $\beta\gamma$ subunits of G proteins, facilitated by adaptor/regulators p101 and p84/87 tethering the enzyme to the plasma membrane.¹³ Interestingly, PI3K γ is not only acting as a kinase but also possesses a scaffolding activity that controls the activation of the phosphodiesterase 3B, thus linking PI3K γ to the modulation of intracellular cAMP levels.^{14,15}

Principally, PI3K γ functions as a chemokine sensor guiding leukocyte migration.¹³ Recent evidence indicates a role in the control of cardiac contractility^{15,16} and in modulating the response of vascular cells to shear stress^{17,18} and inflammation.¹⁹ However, whether PI3K γ is implicated in neovascularization remains unknown.

This study aimed to determine the impact of disrupting the *PI3K γ* gene or PI3K γ catalytic activity on reparative angiogenesis and arteriogenesis in a murine model of unilateral limb ischemia. We also evaluated whether EPC from *PI3K γ* mutant mice have functional alterations that may compromise vasculogenesis.

Methods

For detailed methods, please see the supplemental materials (available online at <http://atvb.ahajournals.org>).

Animal Models

Unilateral hindlimb ischemia was induced in male age-matched *PI3K γ ^{-/-}*, *PI3K γ ^{KD/KD}*, and wild-type (WT) mice.²⁰ Postischemic recovery of limb blood flow (BF) was assessed by laser Doppler flowmetry (Perimed).²⁰ Capillary and arteriole densities, EC proliferation (BrdU incorporation), apoptosis (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling [TUNEL] assay), and presence of monocytes and cKit positive progenitor cells were evaluated in limb muscles as described.²⁰⁻²²

Western Blot Analysis

Western blot analyses for PI3K γ , phospho-Ser473-Akt, total Akt, phospho-Ser1177-eNOS, total eNOS, phospho-Erk1/2, and total Erk were performed in muscle extracts and in EPCs obtained from *PI3K γ ^{-/-}*, *PI3K γ ^{KD/KD}*, and WT mice.

FACS Analysis of Mononuclear Cells

Mononuclear cells (MNCs) from peripheral blood (PB) and BM were analyzed for the expression of cKit, Sca-1 and lineage antigens by flow cytometry (FACS Calibur).

EPC Function

EPC outgrowth from BM-MNCs was determined. EPCs were recognized as adherent cells, taking up acetylated low density lipoproteins (acLDL) and binding *Bandeirea simplicifolia* agglutinin. In addition, EPCs were analyzed for the expression of CD31, flk-1, and VE-cadherin by flow cytometry.

The expression of PI3K γ by WT EPCs exposed to a period of hypoxia or normoxia was evaluated by immunocytochemistry and Western blot. Furthermore, the impact of PI3K γ mutations on the Akt/FOXO1/eNOS axis was evaluated by immunocytochemistry and Western blot.

Apoptosis, migratory and invasive/integrative capacities, as well as NO production by EPCs were analyzed.

Distribution of Injected lin⁻ BM-MNC

Lineage-depleted BM-MNCs from PI3K $\gamma^{-/-}$ and WT donor mice were labeled with 2 different fluorescence tracers, mixed together, and injected into the tail vein of ischemic PI3K $\gamma^{-/-}$ or WT mice. After 3 days, the presence of tracer-labeled cells in PB, spleen, and muscles of recipient mice was analyzed by flow cytometry.

Statistical Analysis

All results are expressed as means \pm SEM. Two-way ANOVA was performed to test for interaction between strain and ischemia. Differences between groups were determined using paired or unpaired *t* test. A probability value of less than 0.05 was considered statistically significant.

Results

For supplemental figures, please see the supplemental materials (available online at <http://atvb.ahajournals.org>).

Effect of Ischemia on Muscular PI3K γ , pAkt, and pErk

In WT muscles, PI3K γ protein, detectable under basal conditions, was upregulated by ischemia (supplemental Figure 1a). As expected, no PI3K γ expression was detected in PI3K $\gamma^{-/-}$ muscles. The absence of PI3K γ translated into reduced Akt phosphorylation in adductor muscles before and after ischemia (supplemental Figure 1b). In contrast, Erk1/2 phosphorylation was not different in the two genotypes (supplemental Figure 1c).

PI3K γ Deletion Impairs Neovascularization and Blood Flow Recovery

The typical capillarization response to ischemia was suppressed in PI3K $\gamma^{-/-}$ gastrocnemius (Figure 1A) and adductor muscles (data not shown, $P<0.02$ versus WT). There was no genotype-related difference in the capillarity of contralateral muscles (Figure 1A). Arteriole density was reduced in the ischemic gastrocnemius and adductor of PI3K $\gamma^{-/-}$ mutants (2.2 ± 0.7 versus 5.3 ± 0.6 art/mm² and 0.7 ± 0.3 versus 1.5 ± 0.3 art/mm² in WT, respectively; $P<0.05$ for both comparisons). Because of the impairment in collateral formation, BF recovery was delayed in PI3K $\gamma^{-/-}$ compared with WT mice (Figure 1B). Histological evaluation of PI3K $\gamma^{-/-}$ ischemic muscles showed hyalin degeneration of myocytes, which

appeared numerically reduced, enlarged, and surrounded by collagen (supplemental Figure IIa).

Immunohistochemistry for leukocyte markers, CD18 and F4/80, revealed increased infiltration of inflammatory cells in *PI3K γ ^{-/-}* ischemic muscles (126 ± 19 versus 71 ± 19 CD18-positive cells per microscopic field in WT, $P=0.06$; 164 ± 21 versus 83 ± 16 F4/80-positive cells in WT, $P<0.01$). Inflammatory cells were distributed across the whole section. This was at variance with WT muscles, in which MNCs typically lined around and within the arteriole wall, with some of them being positive for the progenitor cell marker cKit and the proliferation marker PCNA (supplemental Figure IIb). Independently of the genotype, contralateral muscles did not show any structural alteration or signs of inflammation (data not shown).

Kinase Dead Mutation Does Not Alter Postischemic Neovascularization

In *PI3K γ ^{KD/KD}* mice, the neovascularization response to ischemia was preserved at capillary (Figure 2A) and arteriole level (1.5 ± 0.3 and 1.6 ± 0.4 art/mm², in WT, $P=N.S.$). Consistently, at 2 weeks postischemia, blood flow was restored in *PI3K γ ^{KD/KD}* to the levels observed in WT mice (Figure 2B).

PI3K γ Deletion Impairs Endothelial Cell Proliferation and Survival

The number of BrdU-positive capillaries in *PI3K γ ^{-/-}* ischemic gastrocnemius muscles was half the figure observed in WT muscles ($P<0.01$) (supplemental Figure III). The percentage of TUNEL-positive apoptotic capillaries was increased by 2.4-fold in *PI3K γ ^{-/-}* mice (supplemental Figure IV). In contrast, microvascular apoptosis was not augmented in *PI3K γ ^{KD/KD}* mutants (32.5 ± 1.2 versus $25.6 \pm 2.1\%$ in WT, $P=NS$).

PI3K γ Is Expressed in BM-Derived EPCs and Modulated by Hypoxia

As shown in Figure 3A (left), we found that PI3K γ is expressed by EPCs enriched from WT BM-MNCs. This was confirmed by Western blot analysis (data not shown). Importantly, hypoxia increased the number of PI3K γ -expressing EPCs, as shown by the column graph and the representative microscopy fields in Figure 3A. By flow cytometry, we confirmed that these progenitor cells typically coexpress endothelial markers (CD31, flk-1, and VE cadherin) and incorporate acLDL (Figure 3B).

PI3K γ Deletion Affects the Abundance of Progenitor Cells

Under basal conditions, $\text{lin}^- \text{Sca-1}^+$ cells were more frequent in the BM of *PI3K γ ^{-/-}* mice ($P<0.001$ versus WT), whereas the number of $\text{lin}^- \text{cKit}^+$ and $\text{Sca-1}^+ \text{cKit}^+$ progenitor cells was similar in *PI3K γ ^{-/-}* and WT BM. After limb ischemia, no difference in the content of any studied progenitor population was observed between *PI3K γ ^{-/-}* and WT mice (supplemental Figure Va).

FACS analysis of PB mononuclear cells showed increased abundance of $\text{lin}^- \text{Sca-1}^+$ cells in ischemic *PI3K γ ^{-/-}* mice ($P<0.05$ versus WT, supplemental Figure Vb). In contrast, $\text{cKit}^+ \text{Sca-1}^+$ (supplemental Figure Vb) and $\text{cKit}^+ \text{flk1}^+$ cells (data not shown) were similarly represented in the PB of the 2 genotypes.

PI3K γ Deletion Impairs EPC Function and Survival

As shown in Figure 4A (left), EPC outgrowth from *PI3K γ ^{-/-}* BM-MNCs was strikingly decreased ($40.0 \pm 14.1\%$ of WT, $P<0.02$). In contrast, outgrowth of *PI3K γ ^{KD/KD}* EPC was not altered ($76.0 \pm 14.7\%$ of WT, $P=NS$).

PI3K $\gamma^{-/-}$ EPCs showed impaired migratory response to stromal cell-derived factor-1 (SDF-1) ($P<0.05$ versus WT; Figure 4A, right), but responded properly to VEGF-A ($P=0.98$ versus WT, data not shown). In contrast, no migratory deficit was observed in *PI3K* $\gamma^{KD/KD}$ EPC ($P=NS$ versus WT). Both *PI3K* $\gamma^{-/-}$ and *PI3K* $\gamma^{KD/KD}$ EPCs had impaired capacity to integrate into endothelial network structures in vitro (Figure 4B).

We then evaluated the phosphorylation state of Akt in mutant EPCs. As shown by Figure 5A, we found that, after hypoxia, pAkt-containing EPCs were reduced in *PI3K* $\gamma^{-/-}$ mice. In separate experiments, we found similarly reduced pAkt levels also in *PI3K* $\gamma^{KD/KD}$ (58 ± 5 versus $72\pm5\%$ of total EPCs in WT, $P<0.05$). Western blot confirmed reduced levels of pAkt in hypoxic *PI3K* $\gamma^{-/-}$ and *PI3K* $\gamma^{KD/KD}$ EPC (supplemental Figure VIa).

As disturbed PI3K-Akt signaling is expected to alter eNOS activity, we evaluated the phosphorylation state of eNOS and NO production in mutant EPCs. Western blot showed induction of eNOS phosphorylation at Ser1177 by hypoxia in WT (2.4 ± 0.4 -fold of normoxia, $P<0.05$) but not in *PI3K* $\gamma^{-/-}$ EPCs (1.2 ± 0.3 -fold of normoxia, $P=NS$; supplemental Figure VIb). Furthermore, in *PI3K* $\gamma^{-/-}$ EPCs, the total eNOS/GAPDH ratio was reduced either in normoxic and hypoxic conditions (by 70 and 77% compared with WT EPCs, respectively, $P<0.05$). Intriguingly, peNOS levels were increased in *PI3K* $\gamma^{KD/KD}$ EPCs under normoxia ($P<0.05$ versus WT), but not responsive to hypoxia ($P=NS$ versus normoxia). There was no difference in the levels of total eNOS protein between *PI3K* $\gamma^{KD/KD}$ and WT.

In *PI3K* $\gamma^{-/-}$ EPCs, NO production was significantly reduced (by $70\pm10\%$ as compared with WT EPCs, $P<0.05$). A milder reduction in NO production was observed in *PI3K* $\gamma^{KD/KD}$ EPCs ($30\pm4\%$). Importantly, pretreatment of *PI3K* $\gamma^{-/-}$ EPCs with the NO donor Glyco-SNAP increased their migratory capacity toward SDF-1 $\times 1.6$ folds as compared with the NO donor vehicle ($P<0.05$).

We then evaluated whether mutant EPCs are more prone to apoptosis. FOXO1, a proapoptotic transcription factor that is inactivated by Akt phosphorylation via shuttling from the nucleus to the cytoplasm, was expressed with higher frequency at nuclear level in *PI3K* $\gamma^{-/-}$ EPCs ($33.0\pm8.0\%$ of total cells) as compared with *PI3K* $\gamma^{KD/KD}$ ($8.6\pm3.3\%$) or WT EPCs ($7.3\pm2.1\%$, $P<0.01$ for both comparisons). Furthermore, *PI3K* $\gamma^{-/-}$ EPCs expressed higher levels of the apoptotic marker cleaved caspase-3 after hypoxia (Figure 5C). In separate experiments, *PI3K* $\gamma^{KD/KD}$ EPCs showed similar abundance of cleaved caspase-3 after hypoxia ($3.2\pm1.7\%$) as WT EPC ($2.5\pm1.1\%$, $P=NS$).

Fate of Transplanted Progenitor Cells

Finally, we performed transplantation experiments to confirm the reduced viability of *PI3K* $\gamma^{-/-}$ progenitor cells. To this aim, WT or *PI3K* $\gamma^{-/-}$ BM lin⁻ progenitors were i.v. injected into WT or *PI3K* $\gamma^{-/-}$ recipients (supplemental Figure VIIa). Compared with WT cells, transplanted *PI3K* $\gamma^{-/-}$ cells were less abundant in the PB and more abundant in the spleen (supplemental Figure VIIb), irrespective of the fact that recipients were WT or *PI3K* $\gamma^{-/-}$ mice, thus indicating *PI3K* $\gamma^{-/-}$ cells are prone to rapid destruction. We could not detect donor cells in WT or *PI3K* $\gamma^{-/-}$ ischemic muscles.

Discussion

The implication of PI3Ks and downstream pathways Akt/eNOS and Akt/FOXO/eNOS in angiogenesis, arteriogenesis, and vasculogenesis is well established.⁴⁻¹⁰ Nevertheless, the individual contribution of various PI3K isoforms remains largely unknown. Cellular and ligand specificity suggests specialization of class I isoenzymes. At variance with PI3K α and

PI3K β , which are ubiquitously expressed, PI3K δ and PI3K γ are mainly restricted to the hematopoietic system, but PI3K γ is also detected in cardiomyocytes and endothelial cells (ECs).^{15,16,18,19} Furthermore, PI3K γ is activated by the engagement of G protein-coupled receptors with chemokines, such as SDF-1, which exert stimulatory effects on endothelial growth and EPC recruitment.^{23,24} Thus, PI3K γ might play a crucial role in chemokine-dependent neovascularization. Seminal evidence in support of this possibility was provided by a study showing increased tumoral angiogenesis in mice deficient for the PIP₃-deactivating phosphatase *PTEN*, which was partially rescued by additional disruption of *PI3K γ* .²⁵

The present study newly demonstrates that ischemia-induced angiogenesis is altered in *PI3K γ ^{-/-}* muscles. Incorporation of BrdU by capillary ECs was strikingly reduced during the mounting phase of neovascularization, which was paralleled by 3-fold augmented apoptosis. These data suggest that PI3K γ modulates the capillarization of ischemic muscles, by controlling the balance between EC proliferation and death. We also observed a delayed postischemic recovery in *PI3K γ ^{-/-}* mice and reduced arteriole density in their adductor muscles, a result which supports contribution of PI3K γ to ischemia-induced arterial remodelling. MNCs invading the arterial wall reportedly play a pivotal role in arteriogenesis.²⁶ Arterioles of WT muscles were indeed surrounded and infiltrated by proliferating MNCs and cKit⁺ progenitor cells. In contrast, this pattern was subverted in *PI3K γ ^{-/-}* muscles, which showed diffuse infiltration of inflammatory cells within a network of severely damaged myocytes, but no evidence of MNCs or cKit⁺ progenitors concentrated around arterioles. Similarly, after aortic banding, *PI3K γ ^{-/-}* hearts manifested cardiac necrosis, which triggered a pronounced reactive inflammatory response.¹⁷

We found lower pAkt levels but normal pErk levels in *PI3K γ ^{-/-}* muscles. This is consistent with previous in vitro studies on bovine ECs, showing that shear stress-induced activation of Erk is not inhibited by expression of a kinase-inactive *PI3K γ* mutant.¹⁸ Based on the above results, we initially hypothesized that PI3K γ might modulate neovascularization through an Akt-dependent mechanism. Surprisingly, however, *PI3K γ ^{KD/KD}* mice showed proper formation of arterial collaterals, allowing for a rapid recovery from ischemia. This indicates that PI3K γ kinase activity may be dispensable for proper vascular repair.

We then asked whether PI3K γ plays a role in the modulation of EPC function. A first hint in support is provided by the finding that PI3K γ is constitutively expressed by WT EPCs and upregulated by hypoxia. Hematopoietic lin⁻Sca-1⁺ cells were increased in the BM of *PI3K γ ^{-/-}* mice, a result which is in keeping with previous reports showing abundance of myeloid cells in hematopoietic tissues of *PI3K γ ^{-/-}* mice.^{13,27} After limb ischemia, we observed a more pronounced mobilization of BM lin⁻Sca-1⁺ cells in *PI3K γ ^{-/-}* mutants compared with WT, however no genotype-related difference was detected with regard to the levels of cKit⁺Flk1⁺ vascular progenitor cells in PB.

These observations prompted us to investigate whether steps subsequent to mobilization might be altered as a consequence of the *PI3K γ* deficit. We found that *PI3K γ ^{-/-}* BM-MNC have reduced capacity to generate EPC. In turn, *PI3K γ ^{-/-}* EPCs showed impaired migratory activity toward SDF-1 (a chemokine implicated in the recruitment of hemangiocytes),²³ limited capacity to incorporate into a vascular network in vitro, and diminished resistance to hypoxia. The survival capacity of *PI3K γ ^{-/-}* progenitor cells was further challenged in vivo under ischemic conditions. After injection in WT or *PI3K γ ^{-/-}* mice, *PI3K γ ^{-/-}* lin⁻ cells were cleared from the circulation and accumulated in the spleen, the organ where damaged circulating cells are retained. On the other hand, we could not detect transplanted cells in ischemic limb muscles, which is in agreement with clinical studies showing very low levels of incorporation after bolus injection of BM-derived MNCs.²⁸ Furthermore, our data show

that there is an increased influx of inflammatory cells in *PI3K γ ^{-/-}* ischemic muscles, but less cKit⁺ cells and less neovascularization in comparison to WT animals. The source of cKit⁺ cells invading ischemic muscles remains undetermined. We cannot exclude that, besides controlling the function of BM-derived EPCs, PI3K γ may play a role in the activation of resident progenitor cells, which are increasingly considered for supporting neovascularization.^{29,30} Although we could not detect homing of lin⁻ progenitors to hindlimb muscle, this does not contradict the principle of post-natal vasculogenesis mediated by BM-derived progenitor cells. In vivo, vascular progenitor cells are activated within the BM, followed by mobilization into the bloodstream. Intravenous injection of lin⁻ BM progenitors, as was performed in our model as well as numerous other studies, bypasses this activation in the BM, which is crucial to render cells capable of homing into ischemic tissues.²³ We therefore think that no conclusion can be drawn from our results about the homing capacity of *PI3K γ ^{-/-}* progenitors, the data rather indicate an impaired survival capacity of those cells. Future studies will have to address the role of PI3K γ in activation and liberation of vascular progenitor cells, as well as modulation of homing mechanisms.

Established evidence indicates that PI3Ks regulate EPC functions and survival through phosphorylation/activation and nuclear trafficking of Akt^{8,9} and subsequent phosphorylation/inactivation and nuclear exclusion of FOXO.³¹ In hematopoietic progenitor cells, FOXO1 mediates cell cycle arrest and apoptosis through Fas ligand and the Bcl-2-like-protein Bim.^{31,32} Akt and FOXO converge to eNOS, the former phosphorylating/activating eNOS and the latter repressing eNOS protein expression by binding to the eNOS promoter.⁸ In turn, eNOS modulates EPC mobilization, migration, and survival via the generation of NO.^{10,33} Here, we newly report that *PI3K γ ^{-/-}* EPCs have increased nuclear retention of FOXO1 and impaired modulation of Akt and eNOS phosphorylation after hypoxia. Furthermore, eNOS/GADPH ratio and NO production were reduced in *PI3K γ ^{-/-}* EPCs. Altogether, these results suggest that, in the absence of PI3K γ , the inhibitory control of FOXO by Akt is disrupted, thus accounting for the defective outgrowth from BM-MNCs and increased susceptibility to hypoxia-induced apoptosis of *PI3K γ ^{-/-}* EPCs. Furthermore, decreased NO production seems to play a pivotal role in the migratory deficit, because preincubation of *PI3K γ ^{-/-}* EPCs with an NO donor restored proper motility toward SDF-1. The normal migratory response *PI3K γ ^{-/-}* EPCs to VEGF is compatible with the fact that VEGF tyrosine kinase receptors are associated to PI3Ks different from the isoform γ .

Similar to *PI3K γ ^{-/-}* EPCs, *PI3K γ ^{KD/KD}* EPCs showed defective Akt activation by hypoxia. In *PI3K γ ^{KD/KD}* EPCs, however, FOXO1 was predominantly localized in the cytoplasm, which supports the notion that Akt-independent mechanisms contribute to FOXO relocation/inactivation.^{32,34} In addition, eNOS was found constitutively activated, thus accounting for the almost normal production of NO by *PI3K γ ^{KD/KD}* EPCs and possibly for the absence of overt alterations in outgrowth, survival, and migration. The mechanism responsible for increased eNOS phosphorylation in *PI3K γ ^{KD/KD}* EPCs remains obscure. It is established that kinases different from Akt may contribute to eNOS phosphorylation of a serine residue in the reductase domain (Ser¹¹⁷⁷ in human sequence and Ser¹¹⁷⁹ in bovine sequence).³⁵ In addition, site specific dephosphorylation by PP2A reportedly modulates eNOS activity,¹¹ thereby inducing antiangiogenic effects.³⁶ Compensatory activation of Akt-independent mechanisms or inhibition of PP2A might contribute to maintain high levels of eNOS phosphorylation in *PI3K γ ^{KD/KD}* EPCs.

The differences between null and kinase dead mutations point to the possibility that PI3K γ modulates EPC function and muscular angiogenesis by kinase-independent mechanisms. It was recently shown that several proteins, such as p101, p84, Ras, and phosphodiesterase-3B can bind to PI3K γ , indicating a protein-scaffold function in addition to its kinase activity.¹⁵ Inactivation of PI3K γ scaffolding function results in phosphodiesterase-dependent

accumulation of cAMP,¹⁵ which reportedly exerts detrimental effects on angiogenesis through inhibition of EC proliferation³⁷ and protein kinase A–dependent activation of apoptosis.³⁸

Implications of this study extend to therapeutics. Recently, PI3K γ inhibitors have been introduced with promises of being an “aspirin of the 21st century”.³⁹ One major challenge emerging on the path to developing therapeutically effective inhibitors is to avoid undesired actions on the vasculature of healing tissues.⁴⁰ Drugs able of selectively targeting kinase-dependent or independent functions of PI3K γ may open interesting therapeutic opportunities for the cure of cardiovascular and inflammatory diseases.

Acknowledgments

Sources of Funding

The study was supported by the British Heart Foundation (PG 06/035/20641 and PhD studentship 06/083/21828), the Juvenile Diabetes Foundation Research (grant 1/2004/124), the European community (Marie Curie fellowships to N.K. and A.O., Eugeneheart FP6 IP), the Leducq Foundation, and Regione Piemonte. M.S., P.C., and A.C. are BHF PhD students. C.E. holds a BHF Basic Science Lectureship. The Bristol Heart Institute is associate member of the European Vascular Genomic Network of Excellence (EVGN).

References

1. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med.* 2000; 6:389–395. [PubMed: 10742145]
2. Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res.* 2004; 95:343–353. [PubMed: 15321944]
3. Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem.* 2001; 70:535–602. [PubMed: 11395417]
4. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature.* 1999; 399:601–605. [PubMed: 10376603]
5. Jiang BH, Zheng JZ, Aoki M, Vogt PK. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A.* 2000; 97:1749–1753. [PubMed: 10677529]
6. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res.* 2002; 90:1243–1250. [PubMed: 12089061]
7. Ahmad S, Hewett PW, Wang P, Al-Ani B, Cudmore M, Fujisawa T, Wang P, Haigh JJ, Le Noble F, Mukhopadhyay D, Ahmed A. Direct evidence for endothelial Flt-1 function in nitric oxide-mediated angiogenesis. *Circ Res.* 2006; 99:715–722. [PubMed: 16946136]
8. Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, Kolipara R, DePinho RA, Zeiher AM, Dimmeler S. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest.* 2005; 115:2382–2392. [PubMed: 16100571]
9. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest.* 2001; 108:391–397. [PubMed: 11489932]
10. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med.* 2003; 9:1370–1376. [PubMed: 14556003]
11. Greif DM, Kou R, Michel T. Site specific dephosphorylation of endothelial nitric oxide synthase by protein phosphatase 2A: evidence for crosstalk between phosphorylation sites. *Biochemistry.* 2002; 41:15845–15853. [PubMed: 12501214]
12. Vanhaesebroeck B, Ali K, Bilancio A, Geering B, Foukas LC. Signalling by PI3K isoforms: insights from gene-targeted mice. *Trends Biochem Sci.* 2005; 30:194–204. [PubMed: 15817396]

13. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*. 2000; 287:1049–1053. [PubMed: 10669418]
14. Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R, Wymann MP. Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science*. 1998; 282:293–296. [PubMed: 9765155]
15. Patrucco E, Notte A, Barberis L, Selvetella G, Maffei A, Brancaccio M, Marengo S, Russo G, Azzolino O, Rybalkin SD, Silengo L, Altruda F, Wetzker R, Wymann MP, Lembo G, Hirsch E. PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. *Cell*. 2004; 118:375–387. [PubMed: 15294162]
16. Crackower MA, Oudit GY, Kozieradzki I, Sarao R, Sun H, Sasaki T, Hirsch E, Suzuki A, Shioi T, Irie-Sasaki J, Sah R, Cheng HY, Rybin VO, Lembo G, Fratta L, Oliveira-dos-Santos AJ, Benovic JL, Kahn CR, Izumo S, Steinberg SF, Wymann MP, Backx PH, Penninger JM. Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell*. 2002; 110:737–749. [PubMed: 12297047]
17. Hirsch E, Lembo G, Montrucchio G, Rommel C, Costa C, Barberis L. Signaling through PI3Kgamma: a common platform for leukocyte, platelet and cardiovascular stress sensing. *Thromb Haemost*. 2006; 95:29–35. [PubMed: 16543958]
18. Go YM, Park H, Maland MC, Darley-USmar VM, Stoyanov B, Wetzker R, Jo H. Phosphatidylinositol 3-kinase gamma mediates shear stress-dependent activation of JNK in endothelial cells. *Am J Physiol*. 1998; 275:H1898–H1904. [PubMed: 9815099]
19. Puri KD, Doggett TA, Huang CY, Douangpanya J, Hayflick JS, Turner M, Penninger J, Diacovo TG. The role of endothelial PI3Kgamma activity in neutrophil trafficking. *Blood*. 2005; 106:150–157. [PubMed: 15769890]
20. Emanuelli C, Minasi A, Zacheo A, Chao J, Chao L, Salis MB, Straino S, Tozzi MG, Smith R, Gaspa L, Bianchini G, Stillo F, Capogrossi MC, Madeddu P. Local delivery of human tissue kallikrein gene accelerates spontaneous angiogenesis in mouse model of hindlimb ischemia. *Circulation*. 2001; 103:125–132. [PubMed: 11136697]
21. Emanuelli C, Graiani G, Salis MB, Gadau S, Desortes E, Madeddu P. Prophylactic gene therapy with human tissue kallikrein ameliorates limb ischemia recovery in type 1 diabetic mice. *Diabetes*. 2004; 53:1096–1103. [PubMed: 15047627]
22. Madeddu P, Emanuelli C, Pelosi E, Salis MB, Cerio AM, Bonanno G, Patti M, Stassi G, Condorelli G, Peschle C. Transplantation of low dose CD34+KDR+ cells promotes vascular and muscular regeneration in ischemic limbs. *Faseb J*. 2004; 18:1737–1739. [PubMed: 15345695]
23. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. *Nat Med*. 2006; 12:557–567. [PubMed: 16648859]
24. De Falco E, Porcelli D, Torella AR, Straino S, Iachininoto MG, Orlandi A, Truffa S, Biglioli P, Napolitano M, Capogrossi MC, Pesce M. SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. *Blood*. 2004; 104:3472–3482. [PubMed: 15284120]
25. Hamada K, Sasaki T, Koni PA, Natsui M, Kishimoto H, Sasaki J, Yajima N, Horie Y, Hasegawa G, Naito M, Miyazaki J, Suda T, Itoh H, Nakao K, Mak TW, Nakano T, Suzuki A. The PTEN/PI3K pathway governs normal vascular development and tumor angiogenesis. *Genes Dev*. 2005; 19:2054–2065. [PubMed: 16107612]
26. Ito WD, Arras M, Winkler B, Scholz D, Schaper J, Schaper W. Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. *Circ Res*. 1997; 80:829–837. [PubMed: 9168785]
27. Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, Joza N, Mak TW, Ohashi PS, Suzuki A, Penninger JM. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science*. 2000; 287:1040–1046. [PubMed: 10669416]

28. Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B, Ganser A, Knapp WH, Drexler HE. Monitoring of bone marrow cell homing into the infarcted human myocardium. *Circulation*. 2005; 111:2198–2202. [PubMed: 15851598]
29. Ziegelhoeffer T, Wagner S, Fernandez B, Helisch A, Martin S, Tribulova S, Kuziel WA, Bachmann G, Schaper W. Collateral artery growth (arteriogenesis) after experimental arterial occlusion is impaired in mice lacking CC-chemokine receptor-2. *Circ Res*. 2004; 94:671–677. [PubMed: 14963007]
30. Khmelevski E, Becker A, Meinertz T, Ito WD. Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation. *Circ Res*. 2004; 95:E56–E64. [PubMed: 15331452]
31. Urbich C, Knau A, Fichtlscherer S, Walter DH, Bruhl T, Potente M, Hofmann WK, de Vos S, Zeiher AM, Dimmeler S. FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells. *Faseb J*. 2005; 19:974–976. [PubMed: 15824087]
32. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*. 1999; 96:857–868. [PubMed: 10102273]
33. Chen YH, Lin SJ, Lin FY, Wu TC, Tsao CR, Huang PH, Liu PL, Chen YL, Chen JW. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms. *Diabetes*. 2007; 56:1559–1568. [PubMed: 17389326]
34. Vogt PK, Jiang H, Aoki M. Triple layer control: phosphorylation, acetylation and ubiquitination of FOXO proteins. *Cell Cycle*. 2005; 4:908–913. [PubMed: 15917664]
35. Partovian C, Zhuang Z, Moodie K, Lin M, Ouchi N, Sessa WC, Walsh K, Simons M. PKC α activates eNOS and increases arterial Blood flow in vivo. *Circ Res*. 2005; 97:482–487. [PubMed: 16081872]
36. Urbich Reissner A, Chavakis E, Dernbach E, Haendeler J, Fleming I, Zeiher AM, Kaszkin M, Dimmeler S. Dephosphorylation of endothelial nitric oxide synthase contributes to the anti-angiogenic effects of endostatin. *FASEB J*. 2002; 16:706–708. [PubMed: 11978735]
37. Favot L, Keravis T, Holl V, Le Bec A, Lugnier C. VEGF-induced HUVEC migration and proliferation are decreased by PDE2 and PDE4 inhibitors. *Thromb Haemost*. 2003; 90:334–343. [PubMed: 12888882]
38. Kim S, Bakre M, Yin H, Varner JA. Inhibition of endothelial cell survival and angiogenesis by protein kinase A. *J Clin Invest*. 2002; 110:933–941. [PubMed: 12370271]
39. Ruckle T, Schwarz MK, Rommel C. PI3Kgamma inhibition: towards an ‘aspirin of the 21st century’? *Nat Rev Drug Discov*. 2006; 5:903–918. [PubMed: 17080027]
40. Doukas J, Wrasidlo W, Noronha G, Dneprovskaya E, Fine R, Weis S, Hood J, Demaria A, Soll R, Cheresch D. Phosphoinositide 3-kinase gamma/delta inhibition limits infarct size after myocardial ischemia/reperfusion injury. *Proc Natl Acad Sci U S A*. 2006; 103:19866–19871. [PubMed: 17172449]

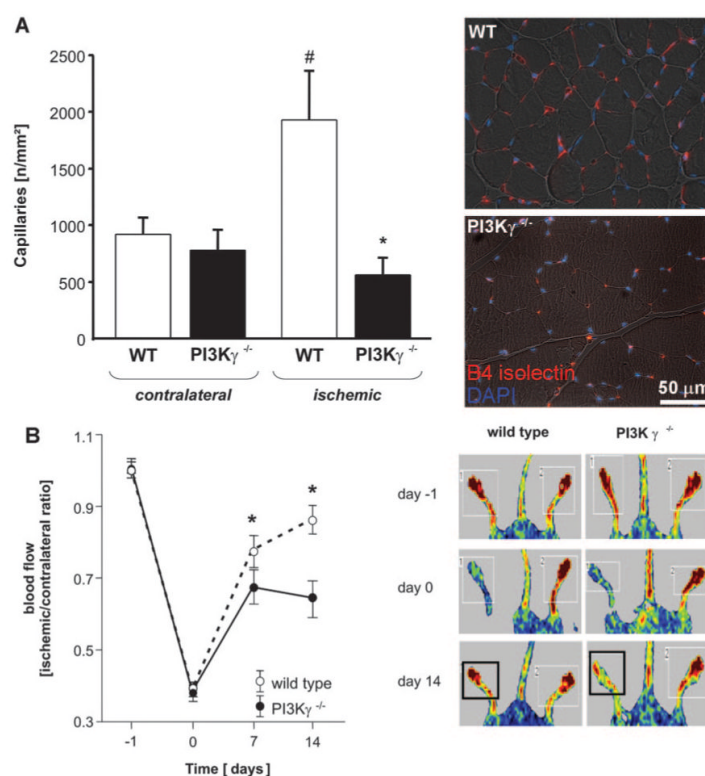


Figure 1.

Deletion of PI3K γ impairs neovascularization and reperfusion. A, Capillary density of ischemic and contralateral gastrocnemii (left). Representative microphotographs are shown in the right panel. B, Blood flow recovery (left) and representative laser Doppler images (right). Values are means \pm SEM. * P <0.05 vs WT and # P <0.05 vs contralateral.

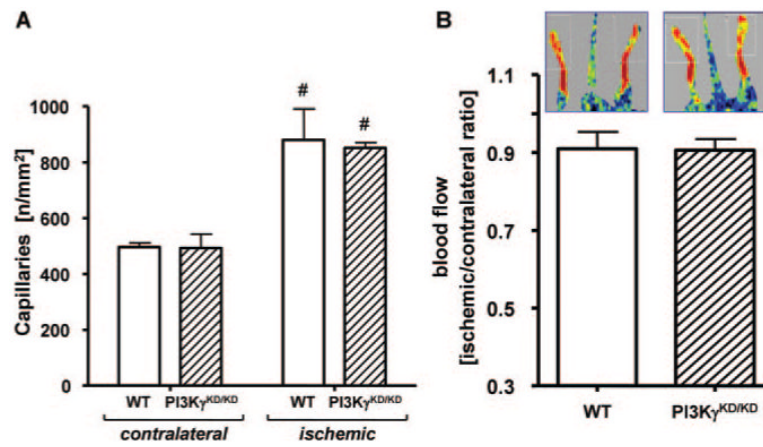
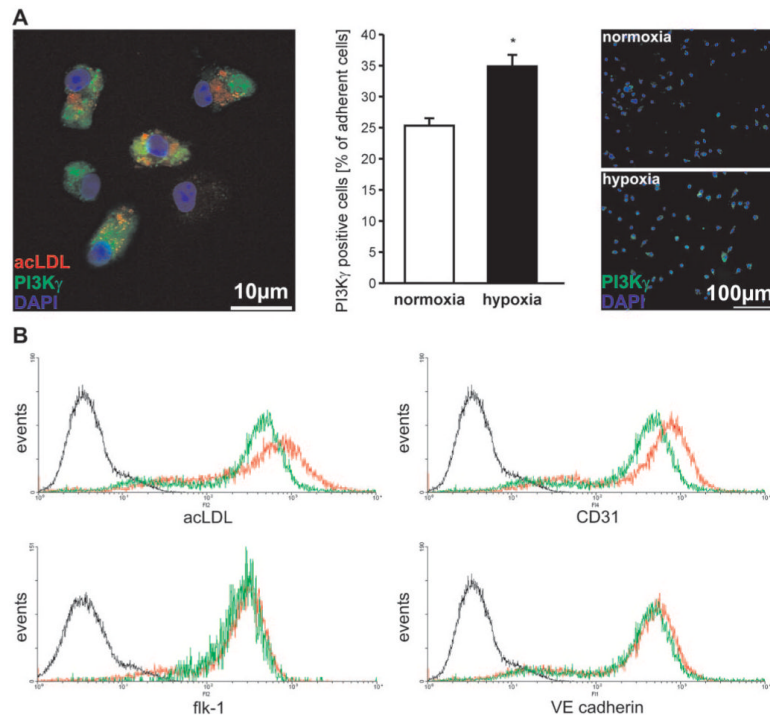


Figure 2.

Kinase dead mutation does not alter reparative neovascularization. A, Capillary density of ischemic and contralateral gastrocnemius muscle. [#] $P < 0.05$ vs contralateral. B, Blood flow recovery and representative laser Doppler images at 2 weeks from induction of ischemia.

**Figure 3.**

WT EPC express P I3K γ . A, PI3K γ -positive (green) and acLDL uptaking (red) EPCs. Nuclei appear blue. Hypoxia increases the number of PI3K γ -positive EPCs (middle and right). * $P < 0.05$ vs normoxia. B, EPC characterization by flow cytometry (green and red represent 2 independent experiments, black negative control).

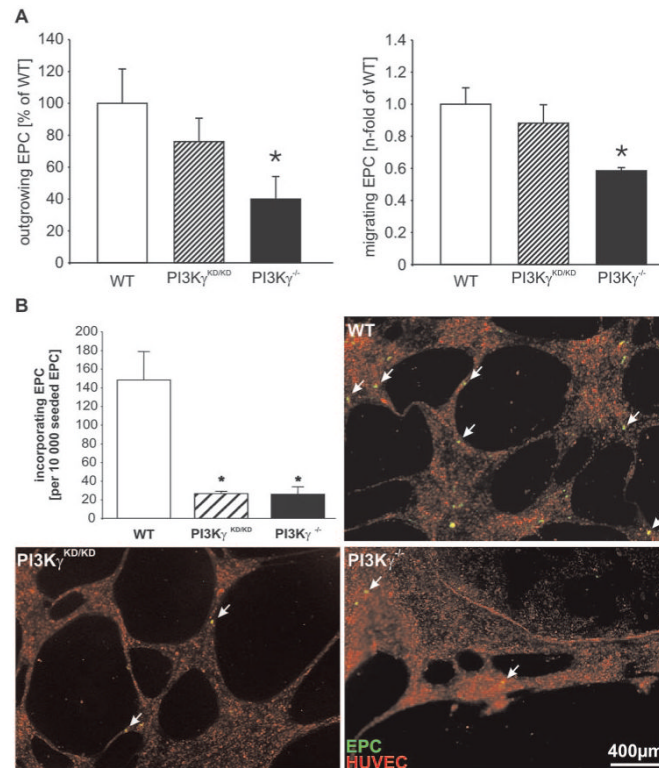


Figure 4.

PI3K γ deletion impairs EPC function. A, PI3K γ deletion impairs EPC outgrowth (left) and migration toward SDF-1 (right). B, Both PI3K γ deletion and kinase dead mutation reduce the rate of EPC incorporation into vascular networks in vitro. EPCs appear yellow (pointed by arrows). * $P < 0.05$ vs WT.

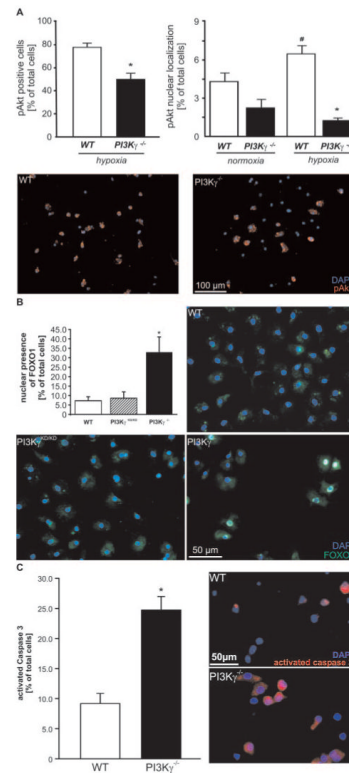


Figure 5.

Reduced pAkt levels and increased apoptosis markers in PI3K γ ^{-/-} EPC. A, Abundance (left) of pAkt-positive BM-EPC and nuclear localization (right) of pAkt. Representative images are shown (bottom). * $P < 0.05$ vs WT, # $P < 0.05$ vs normoxia. PI3K γ ^{-/-} EPC exhibit a higher frequency of FOXO1 (panel B) and caspase-3 positivity (panel C).